

Interim Protocol for Measuring Microbial
Transformation Rate Constants for Suspended
Bacterial Populations in Aquatic Systems

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INTERIM PROTOCOL FOR MEASURING MICROBIAL TRANSFORMATION
RATE CONSTANTS FOR SUSPENDED BACTERIAL POPULATIONS
IN AQUATIC SYSTEMS

by

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FOREWORD

As environmental controls become more expensive and penalties for judgment errors become more severe, environmental management requires more precise assessment tools based on greater knowledge of relevant phenomena. As a part of this Laboratory's research on occurrence, movement, transformation, impact and control of chemical contaminants, the Measurements Branch determines the occurrence of unsuspected organic pollutants in the aquatic environment and develops and applies techniques to measure physical, chemical, and microbial transformation and equilibrium constants for use in assessment models and for development of property reactivity correlations.

Regulatory activities by the EPA's Office of Toxic Substances require information about the environmental fate of organic chemicals. Part of this information is provided through research to develop microbial transformation rates for chemicals in freshwater. This report presents an interim protocol for performing this research to help assure that data developed by different laboratories or researchers, or by the same researcher at different times, are produced in a consistent, comparable manner. The protocol will be subject to change as new information is developed in continuing studies of the environmental factors affecting microbial degradation.

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ABSTRACT

This report represents an interim protocol for performing research to measure microbial degradation rates of organic chemicals in freshwaters. For chemicals, a major transformation pathway influencing their environmental fate is microbial degradation. The interim protocol presented provides a basis for measurement of microbial degradation rates such that reliable, comparable and consistent data can be obtained by different laboratories and research investigations. As additional research and information is gathered on the environmental factors affecting microbial degradation by suspended bacterial populations in freshwater, the outlined protocol will be modified. The Office of Toxic Substances is required to estimate the environmental fate of many organic chemicals. This measurement procedure provides the OTS with a reliable and consistent set of procedures through which reliable predictions of microbial degradation rates can be made in aquatic systems.

INTRODUCTION

Under the Toxic Substances Control Act of 1976 (PL 94-469), EPA's Office of Toxic Substances is required to review the potential risk to human health and the environment of new chemicals before manufacture and use are permitted. In the two components of risk, exposure and hazard, exposure to chemicals in the environment is dependent on the chemical's environmental fate. For many chemicals, a major transformation pathway influencing their environmental fate is microbial degradation. Therefore in assessing risk, it is necessary to have some estimate of the microbial transformation rate of each chemical.

The Office of Toxic Substances estimates microbial transformation rates of chemicals proposed for manufacture by comparing each new organic chemical's structure or other known properties with those of chemicals whose microbial transformation rates have been established. Chemicals with similar structures/properties are expected to have similar microbial transformation rates. Unfortunately, microbial transformation rates have been measured for only a few chemicals. Much more experimental data are needed.

The database of measured microbial transformation rates for individual chemicals continues to be expanded as studies are performed to better understand the many environmental factors that influence microbial degradation. Much remains to be learned about the ways in which microbial population density and diversity, accessibility of the chemical substrate to the microcosms, and other factors influence transformation rates.

While these studies continue, it is necessary to provide measured microbial transformation rates for chemicals based on current knowledge to meet regulatory needs. These rates must be measured in a manner

that is reproducible and for which the results of measurement of one chemical can be compared to those of another with confidence, even though the influence of some environmental factors is not fully understood.

The purpose of this report is to present a sampling and measurement protocol that has been applied over several years at the Athens Environmental Research Laboratory. The methodology has been found to provide reproducible second-order rate constants using suspended natural populations in aerobic aquatic systems.

SOURCES OF MICROBIAL POPULATIONS AND SAMPLING PROCEDURES

Freshwater samples to provide natural microbial populations can be taken from a variety of natural sources, including rivers, streams, ponds, and lakes. Investigations by Paris et al. (1983) of approximately 35 different environmental waters from across the continental United States showed no significant differences in transformation rates of chemicals as a function of water source. Biological waste treatment facilities and other sources influenced by high concentrations of substrates should be avoided as sources, however, because they are likely to contain populations whose diversities have been strongly influenced by unnatural substrates and are not typical of populations in ambient freshwaters.

Collection of microbiota is accomplished by a grab sampling technique in which 3.8-liter amber glass jugs are immersed in a freshwater source until full. Samples taken within the vicinity of the analysis laboratory need no temperature control if transported immediately; those requiring transport for more than 2 hours should be cooled on ice. At the time of sampling, water temperature should be taken and recorded in the laboratory notebook for future reference.

When a water source is sampled for the first time, it is advisable to measure nitrogen, phosphorus, total organic carbon, and pH by standard EPA methods (see Methods for Chemical Analysis of Water and Wastes, 1979. EPA-600/4-79-020). This characterization may be useful in determining the cause of any anomalous results that may arise in laboratory measurements.

Population densities should be measured by standard plate counts (Heterotrophic Plate Counts) as outlined in the American Public Health Association's Standard Methods (APHA, 1985). This characterization serves to establish the baseline population density of the ambient water or to establish the need for following an alternative concentration procedure for increasing population densities in order to observe measurable rates in the transformation of test chemicals.

The time lapse between sampling and initiation of the rate constant measurement phases should be no more than 12 hours. Once the microbial samples have been returned to the laboratory, handling prior to experimental rate constant measurement can take two courses.

In one procedure, the sample is used at the natural population density when sampled. The test chemical is added and the chemical's transformation rate is measured.

In the alternative procedure, the natural microbial population is enhanced through a concentration step. This 10:1 concentration step requires larger volumes of source water.

Bacterial populations in water samples are concentrated 10-fold by filtering 10 liters through a membrane filter (0.2 μm pore diameter, Nucleopore or equivalent) that has been prewashed with distilled water. After filtration, filters are placed in a 2-liter, wide-mouth, cotton-plugged

Erlenmeyer flask containing 1 liter of the original source sample.

Sterile, aqueous, concentrated stock solutions of nutrients are prepared to yield concentrations (g/L) of each of the following: NH_4Cl (0.5), $(\text{NH}_4)_2\text{SO}_4$ (0.5), Na_2HPO_4 (0.5), KH_2PO_4 (0.5), MgSO_4 (0.001), and FeCl_3 (0.001). No more than 1 ml of each nutrient is added to the concentrated bacterial population. These bacterial suspensions then are incubated for 48 hours at 22°C in a temperature-controlled shaker (150 to 200 rpm) before each experiment is initiated. This procedure enhances the bacterial population 10- to 100-fold.

BACTERIAL CONCENTRATION MEASUREMENTS

Trypton Glucose Extract Agar, (Difco Laboratories) at a concentration of 24 g/L is used in determining the bacterial concentration by standard techniques (APHA, 1983). For non-sterile treatments, reaction vessels are removed from the incubator/shaker and serial dilutions ranging from 10^{-2} to 10^{-7} are made into autoclaved sterile dilution medium (deionized water or sterile natural water). The vessels then are agitated to suspend the organisms.

From the series of dilutions, pour plates then are prepared. The anticipated population count for the pour plates should span a 2- to 3-fold range (e.g., 10^3 , 10^4 , and 10^5 organisms/ml) to assure representative counts and to encompass the range of total viable organisms in the reaction flasks. Each dilution is transferred in duplicate or triplicate to 100 X 15 mm, disposable petri dishes (Fisher Scientific). To each plate, 5 to 7 ml of Trypton Glucose Extract Agar (TGE) is added and mixed thoroughly with the sample. Plates then are incubated for 48 hours at $22 \pm 1^\circ\text{C}$ in the temperature-controlled incubator/shaker. Following the

48-hour incubation, plates are removed for bacteria counting. Bacterial colonies are counted for each plated dilution (30 to 300 colonies), using a Quebec colony counter or other suitable counting instrument, tabulated and averaged.

Each time bacterial concentrations are measured, four sterile controls (two filter-sterilized and two autoclaved) are plated by transferring 1-ml volumes to plates followed by addition of the TGE. Control plates must be incubated with and counted within the same period as non-sterile reaction vessel enumerations. As an additional control, a sterile agar control is prepared by pouring TGE into petri dishes followed by incubation under the above conditions.

Data are recorded as the average of duplicate or triplicate plates with measurements listed as colony-forming units (CFU) per liter or as number of organisms per liter, total viable count. For each time-dependent sampling for chemical disappearance, plate count analyses should be performed. Procedures for use of the resulting plate count averages are discussed under the section entitled "Data Compilation Methods."

TEST CHEMICALS

Test chemical solutions are prepared by dissolving known amounts of chemical into filter-sterilized, deionized water at concentrations not exceeding 50 percent of the compound's water solubility. Solutions are then filter-sterilized through sterile 0.2- μ m membrane filters. This filtrate serves as the stock solution from which 1- to 2-ml volumes are added to reaction flasks containing environmental waters to yield concentrations between 0.1 and 10 ppm. Should the solubility of the test chemical be such that adequate aqueous concentrations are difficult

to achieve, appropriate solvents are used to make concentrated stock solutions from which volumes of less than one 1 ml can be added to reaction flasks to obtain the appropriate test chemical concentrations.

Abiotic (Sterile) Controls

Sterile controls are prepared by two methods to assess potential abiotic influences on measured microbial rate constants. Any loss of chemicals within these control flasks can be attributed to abiotic (chemical) transformation and appropriate correction made to non-sterile microbiological treatments.

The first method consists of filtration of one liter of the non-sterile aquatic sample through a glass fiber filter to remove coarse debris, followed by filtration through 0.22- μ m presterilized filters. The second method consists of removing coarse debris (sticks, leaves, etc.) as in the method above, followed by dispensing 200 ml of aquatic sample into 250-ml reaction flasks in duplicate, stoppering the flasks loosely and autoclaving (steam sterilizing) them at 15 psi at 121°C for 20 minutes. Following steam sterilization, samples are cooled to ambient room temperature and placed in an incubator/shaker for 48 hours before addition of the test chemical. This allows for equilibration of sample and population changes.

Biotic Treatment of Test Chemical

Samples are prepared for biotic treatment by adding 200 ml of the aquatic sample to each of four 250-ml Erlenmeyer flasks or 300 ml of sample to each of four 500-ml Erlenmeyer flasks, depending on the number of anticipated samples to be withdrawn during treatment. Appropriate volumes of the test chemical solution are added to produce concentrations in two flasks that are 10 to 100 times the concentrations in the other two flasks (i.e. two flasks

at 0.1 ppm and two flasks that are 10.0 ppm). Neither concentration should be below 0.1 ppm or above 10.0 ppm.

The purpose of using two concentrations is to assure that a sufficient percentage of the test chemical degrades within a 3-week maximum treatment time to permit precise measurement of the rate constant (low concentration) without degrading too rapidly for precise measurement of rate (high concentration). Ideally 75 percent of the chemical should degrade between 72 hours and 504 hours (3 weeks). If the experiment must be repeated, a single concentration based on the initial observations can be used. Additional discussion of kinetics is presented in the data compilation methods section.

Incubation and Temperature Control

Following preparation of samples and spiking to desired concentrations with the test chemical, reaction vessels are transferred to an environmentally controlled temperature/incubator equipped with a gyratory shaker. Samples are incubated under shake conditions (150 - 200 rpm) and held under a constant temperature, of $20^{\circ}\text{C} \pm 2^{\circ}\text{C}$. At predetermined intervals, reaction flasks are sampled in the the incubator/shaker and samples are withdrawn for gas chromatographic (solvent extraction) or high performance liquid chromatographic analysis using sterile aseptic techniques.

DATA COMPILATION METHODS

Test Chemical

Raw data on the test chemical are obtained by measuring the concentration of chemical remaining in the reaction flask at specific time intervals. The measured concentration at time zero serves as the reference point for the remaining points. Data are processed using one

of two methods. The first method consists of plotting, on semi-log paper, the Ln (natural log) of the concentration of the parent chemical remaining on the Y-axis and the time of sampling (days, hours) on the X-axis. The slope of the line, assuming that first-order conditions have been met, is the first-order slope (time^{-1}). The second method consists of processing data using the Lotus 1-2-3 software with an IBM PC/XT or other computer. Software programs for slope are based on the linear least squares analysis of the Ln of parent chemical concentration remaining versus time. Outputs from the linear regression program are (1) slope (pseudo-first-order rate constant), (2) Y-intercept, (3) variance, (4) standard deviation (SD) of Y-intercept, (5) standard deviation (SD) of the slope, (6) the correlation coefficient (r^2), and (7) second-order rate constant (k_b , $\text{Liters org}^{-1} \text{ hr}^{-1}$).

Bacterial Population Enumeration

Bacterial population enumerations are made concurrently with each measurement of test chemical concentration remaining. Raw data consist of concentration measurements of bacteria per milliliter or per liter at each time sampling point. The mean bacterial concentration is calculated for the entire transformation period. This mean value is used in calculating the second-order microbial degradation rate constant.

Calculation of Second-order Rate Constants

Following determination of the first-order rate constant for chemical disappearance (k , hr^{-1}), second-order rate constants are calculated by dividing the pseudo-first-order rate constant by the average bacterial concentration (CFU or organisms per liter). By following both parameters under controlled conditions (pH, temperature, etc.), second-order rate

constants can be obtained for a number of chemicals using natural aquatic systems.

The utility of this protocol has been demonstrated by comparative determinations of the second-order microbial rate constants of standard reference chemicals (unpublished report). Two standard reference chemicals (methyl ester of 2,4-dichloro phenoxy acetic acid and propanil) were investigated using natural pond water over a period of 1 year. Second-order rate constants ranged from 7.8×10^{-9} to 9.2×10^{-9} l org⁻¹ hr⁻¹ for nine determinations with the methyl ester of 2,4-D. Propanil yielded rate constants ranging from 1.1 to 6.4×10^{-11} l org⁻¹ hr⁻¹ for six determinations. Coefficients of variation were 32 to 72% for the methyl ester of 2,4-D and 42 to 69% for propanil, both similar to the 65% observed by Paris et al. (1983). No seasonal effects were observed. Use of a standard reference chemical directly aids the investigator in establishing his confidence in the protocol measurements and allows for interlaboratory comparative investigations in application of the protocol.

The applicable form of the second-order rate expression used throughout this interim protocol is:

$$-ds/dt = k_b[B_T][S_T]$$

where: k_b = Second-order rate constant (l org⁻¹ hr⁻¹)
 $[B_T]$ = Measured bacterial concentration (CFU or org per liter)
 $[S_T]$ = Measured substrate (chemical) concentration (mg per liter)

Acclimation/Adaptation

In the measurement of second-order biodegradation of organic chemicals in aquatic systems, adaptation or acclimation periods often are observed

prior to the rapid transformation of some of the test chemical(s). This period is eliminated from the calculations of the second-order rate constant because little transformation of the chemical takes place during this period. In addition, for those chemicals for which hydrolytic or oxidative mechanisms are the dominant mechanisms, adaptation periods are short compared to the overall transformation period. As a result, the period can be reasonably eliminated within the overall calculations and only the first-order disappearance portion of the transformation curve is used to determine the rate constant. Upon visual observation of the degradation curve (i.e. graphical plot of disappearance of chemical concentration versus time) the following procedure for calculation of the first-order slope for chemical (substrate) disappearance is followed. An approximately 15 to 20 percent drop from the initial chemical concentration is taken as the first concentration-time point for calculation of the disappearance slope. From this "initial" concentration-time point disappearance is then followed until 75 percent of the initial concentration has been depleted.

LABORATORY PRACTICES

Methods for measurement of concentrations of test chemicals and metabolites must be carefully and clearly documented selected to provide acceptable detection levels, precision, accuracy, freedom from interference, and speed. Methods most commonly used are gas chromatography and high performance liquid chromatography. Chemical solvents must be of high spectral grade. Extraction procedures must be clearly documented and reported to eliminate discrepancies and to allow for reproducible analysis and experimentation by gas chromatographic methods when used.

FORMAT FOR REPORTING DATA

Aquatic Site

A description of the site and method of sampling must be recorded for each source of microbial population. Appropriate characterization data includes a chemical analysis (N,P,C), pH, microbial density at time of sampling, and temperature at time of sampling.

Test Chemical(s)

Physical/chemical data for test chemicals should include: (1) water solubility (mg/L), (2) molecular weight, (3) Chemical Abstract Service number, (4) chemical source, and (5) purity.

The analytical methodology for the test chemical should be reported in a concise and detailed manner:

Analytical Procedure: _____

(The detailed procedure is described or a reference is given)

Extraction Solvent: _____

Instrumentation Used: GC: _____ HPLC: _____

Detector: _____

Column: _____

Temperature Program: _____

Mobile Phase: _____

Internal Standard: _____

Linear Range of Analysis: _____

Data for Rate Measurement

Chemical Concentrations: The following example serves as a suggested format for recording rate data:

Chemical Name: _____

CAS Number: _____

<u>Time (hr, day, etc.)</u>	<u>Concentration of Chemical Remaining (mg/L, ppm, moles, etc.)</u>
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Data for duplicate or triplicate treatments will be recorded for each time point.

Bacterial Concentrations: Bacterial data will be recorded in a format similar to that described above for each treatment.

Summary Table: For each treatment (including controls) the summary table should consist of the following information.

<u>Chemical Treatment (Concentration) (mg/L, ppm, μmoles, etc.)</u>	<u>First-Order Rate Constant (time⁻¹)</u>	<u>Mean Bacterial Conc. (org. per liter or CFU/liter)</u>	<u>Calculated Second-Order Rate Constant L org⁻¹ hr⁻¹</u>
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ASSUMPTIONS

Several basic assumptions serve as the foundation for measurements of microbial transformation rate constants under this protocol. Use of total viable plate count as a measure of microbial population concentrations provides a measurement that is assumed to be proportional to the number of microorganisms participating in the biodegradation process. For chemicals for which the concept was developed and tested, many of the culturable populations indigenous to aquatic systems support the necessary

constitutive enzymes for microbially-mediated hydrolysis and oxidation reactions. Moreover, as pointed out in the investigations by Paris et al. (1981, 1983), the transformation/biodegradation process is pseudo-first-order with respect to bacterial concentration and; therefore, proportional to the density of total viable bacteria in the system plated on TGE. It is further assumed that the substrate (test chemical) concentration is much less than the theoretical K_s half-saturation concentration and that reaction kinetics are first-order with respect to substrate concentration. It also is assumed that carbon and energy contributions from the chemical under investigation are not sufficient to cause measurable growth of the constitutive populations. The second-order mathematical description of biodegradation serves as a reproducible and reliable measurement of microbial transformation rate constants for organic chemicals in aquatic systems.

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